Genetic Variation in the Premature Aging Gene *WRN*: A Case-Control Study on Breast Cancer Susceptibility

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Abstract

The high risk of developing cancer seen in human genetic diseases that resemble accelerated aging provides support for a tumorigenic contribution of the mechanisms and genes responsible for regulating life span and aging. We therefore speculated that the *WRN* gene (encoding RECQL2, a DNA helicase), the germline mutation of which causes the progeroid disorder Werner syndrome, may be associated with breast tumorigenesis. This hypothesis was tested in this case-control study of 935 primary breast cancer patients and 1,545 healthy controls by examining single-nucleotide polymorphisms (SNPs) in *WRN*. We were also interested in knowing whether any identified association between *WRN* and breast cancer was modified by reproductive risk factors reflecting susceptibility to estrogen exposure. Our hypothesis is that because estrogen is known to promote breast cancer development via its mitogenic effect leading to cell proliferation, and because *WRN* is an essential gene, as its suboptimal function leads to a severe decrease in proliferation, estrogen stimulation may have a protective effect on cells harboring variant *WRN*, allowing them to survive and proliferate for the prolonged period needed for tumor formation. Support for this hypothesis came from the following observations: (*a*) one SNP in *WRN* was significantly associated with breast cancer risk (P = 0.002); (*b*) haplotype and diplotype analyses, based on different combinations of multiple SNPs in *WRN*, revealed a strong association with breast cancer risk; (*c*) this association between risk and putative high-risk genotypes was stronger and more significant in women with a longer interval between menarche and first full-term pregnancy; and (*d*) the protective effect conferred by having a higher number of full-term pregnancy was only significant in women with homozygous or heterozygous wild-type *WRN* genotypes. This study provides support for the tumorigenic role of *WRN* in breast cancer development, suggesting that breast cancer can be driven by the aging associated with variant *WRN*, the tumorigenic contribution of which might be enhanced as a result of increased cell growth due to estrogen exposure. (Cancer Epidemiol Biomarkers Prev 2007;16(2):263–9)

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Introduction

Breast cancer is a major health problem, and the exploration of its causes is an issue of public health importance (1-3). Given that almost all cancers occur in the elderly, it is tempting to speculate that genes regulating the aging process might play a role in tumorigenesis (4, 5). Mutations in the human *WRN* gene, which codes for a DNA helicase, give rise to a rare autosomal recessive genetic disorder, Werner syndrome (6-8). Werner syndrome is a premature aging disease characterized by a predisposition to cancer and early onset of symptoms related to normal aging, including osteoporosis, ocular cataracts, graying and loss of hair, diabetes mellitus, arteriosclerosis, and atherosclerosis (7, 8). The characterization and study of the *WRN* gene suggest that cells derived from patients with *WRN* mutation show increased genomic instability, manifested as chromosomal alterations, and that WRN protein participates in several important DNA metabolic pathways, including DNA replication, recombination, and telomere maintenance to maintain genomic stability, and that its primary function may be in DNA repair (9-11). *WRN* is a caretaker of the genome, and WRN protein represents an important link between defective DNA repair and the processes related to aging and cancer. In the present study, we therefore selected *WRN* as a candidate gene of breast cancer and used the single-nucleotide polymorphisms (SNPs) of this gene to explore its tumorigenic contribution to breast cancer susceptibility. Furthermore, we were interested in knowing whether any association between the *WRN* gene and breast cancer was modified by reproductive risk factors reflecting the level of estrogen exposure or susceptibility to estrogen exposure. The rationale underlying this hypothesis is that because estrogen is known to promote breast cancer development via its mitogenic effect leading to cell proliferation (12), and because *WRN* is an essential gene, as its suboptimal function leads to a severe decrease in proliferation (13), estrogen stimulation may have a protective effect on cells (14) harboring variant *WRN*, allowing them to survive and proliferate for the prolonged period needed for tumor formation. The present study was done to examine this hypothesis.

Materials and Methods

Study Population. Our case-control study is part of an ongoing cooperative study aimed at understanding the causes of breast cancer in Taiwan, which is characterized by low incidence, early tumor onset, hormone dependency, and novel genomic alterations (15-21). Because of the low incidence of breast cancer in the Taiwanese population, which suggests an overall lower effect of common risk factors, and because of its homogenous genetic background, this population is considered to have certain advantages for studying the effects of

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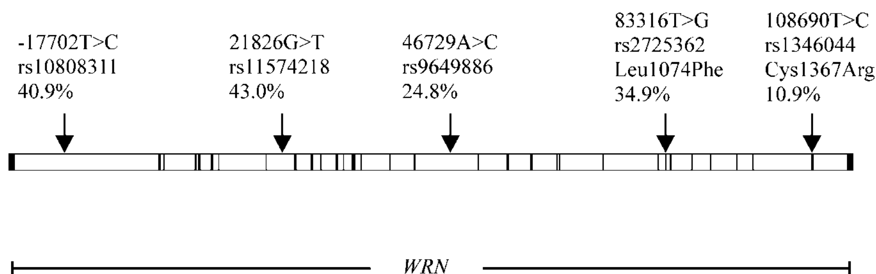

Figure 1. Schematic diagram of the location of the SNPs in *WRN* gene. *Black boxes,* exons of *WRN*; *arrows,* positions of the five SNPs with a minimum allele frequency of 10% used in this study. The percentages indicate the frequency of the variant (rare) allele.

subtle genetic variations (17, 20), such as SNPs. Furthermore, the use of a genetically homogenous population (22) reduces the chance of false positives due to population stratification. We studied 935 female breast cancer patients and 1,545 healthy female controls. All breast cancer patients had pathologically confirmed incident primary breast cancer. All were diagnosed and treated at the Tri-Service General Hospital or the Changhua Christian Hospital between March 2002 and August 2005. These patients accounted for almost all (>90%) women with breast cancer attending our breast cancer clinics during the study period, the remaining patients being excluded because of a lack of suitable blood specimens. No significant differences in breast cancer risk factors were found between the included and excluded women. In addition, because these are two of the major breast cancer clinics in northern and central Taiwan, our patients accounted for a significant proportion (~40%) of all breast cancer cases diagnosed during the study period in these regions. We randomly selected controls from women attending the health examination clinics of the same hospitals during the same period. These women underwent a 1-day comprehensive health examination (including regular breast screening using X-ray mammography and ultrasonic examination), and those showing any evidence of breast cancer, suspicious precancerous lesions of the breast, or other cancers were excluded from the control group. Almost all (>95%) potential controls who were initially identified actually participated in the study, and controls accounted for about 20% of all women attending the clinics, and no significant differences were found in terms of socioeconomic status between those included and those not included.

Considerations regarding methodologic issues in the present study (such as study design, sampling scheme, and potential bias) have been described in detail previously (17, 20, 21), and the validity of our study approach has been addressed and confirmed in these studies.

Data Collection. Informed consent was obtained from all study participants before collection of epidemiologic data through personal interview. The 45-min in-person interview, completed by all study participants, was administered by trained interviewers following institutional guidelines for human subjects. Data on smoking habits, alcohol consumption, and hormone replacement therapy were collected from both cases and controls. Other information, including menstrual and reproductive factors [age at menarche, age at first full-term pregnancy (FFTP), age at menopause, and number of FTPs], family breast cancer history, body mass index, and residence history, was also collected. Menopausal status was assessed at the time of diagnosis. Women with hysterectomy or bilateral oophorectomy were considered as postmenopausal, whereas the very few women with dubious menopausal status were considered as missing data. At the completion of the interview, blood was taken for DNA isolation and molecular analysis. All samples were examined blind by laboratory personnel.

Genotyping. The current data from HapMap, *WRN* in the Chinese population, shows three blocks[8]; in addition, the Taiwanese population is genetically homogenous, and linkage disequilibrium between SNPs is much stronger than that in other populations (17, 20, 22). Thus, we used five SNPs (Fig. 1) in these three blocks to detect genetic variation in this candidate gene. The five SNPs in the *WRN* gene chosen from the HapMap project were rs10808311 (−17702T>C) in the 5′-untranslated region, rs11574218 (21826G>T) in intron 1, rs9649886 (46729A>C) in intron 8, rs2725362 ($Leu^{1074}Phe$, 83316T>G) in exon 21, and rs1346044 ($Cys^{1367}Arg$, 108690T>C) in exon 34 (Fig. 1). These SNPs were chosen because they are evenly distributed throughout the entire gene and have a minor allele frequency of >10% for genotyping. Taqman assays were ordered from Applied Biosystems, Inc. (Foster City, CA). The primers and probes were mixed with PCR reagents in the Taqman assay following the manufacturer's instructions. The plates were sealed and heated at 95°C for 5 min and were subjected to 45 to 50 cycles of 92°C for 15 s and 60°C for 1 min. PCR was carried out in a GeneAmp PCR System 7000 thermocycler (Applied Biosystems). The samples were read and analyzed in an ABI Prism 7000 sequence detection system using version 3.5 software. Each genotyping plate contained positive and negative controls. Tests were repeated on ~10% of the samples to ensure quality control in genotyping, and genotype scoring was done separately by two reviewers to confirm the results.

Statistical Analysis. We followed our previously established sequential steps (17, 21), including a test for Hardy-Weinberg equilibrium (23) and measurement of the relative disequilibrium (D') value to estimate the degree of linkage disequilibrium between individual SNPs (24). Risks were estimated as the odds ratio (OR) and 95% confidence interval (95% CI) using logistic regression models to determine putative high-risk genotypes of *WRN* for breast cancer. Any differences in genotypic frequency of individual SNPs between cases and controls were tested using multiple logistic regression models (25) with simultaneous consideration of known risk factors [age, family history of breast cancer in first- and second-degree relatives, age at menarche, history of FTP (yes versus no), menopausal status (premenopausal versus postmenopausal), and the body mass index] for breast cancer, and adjusted Ps for the association were estimated.

Pairwise linkage disequilibrium between any two alleles of the five polymorphic sites was estimated as the D' from the haplotype data using the equations: (*a*) $D = P_{AB} - P_A P_B$; (*b*) $D' = D/D_{max}$, where $D_{max} = \min(P_A P_b, P_a P_B)$ if $D < 0$; and (*c*) $D' = -D/D_{min}$, where $D_{min} = \max(-P_A P_B, -P_a P_b)$ if $D < 0$, and the statistical significance was evaluated using Fisher's exact test. The distribution of haplotypes in the cases and controls was compared using the χ^2 test. Haplotypes were inferred using the PHASE program available online,[9] which

[8] http://www.hapmap.org/

[9] http://www.stat.washington.edu/stephens/software.html

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reconstructs haplotypes from population genotyping data (26). The highest probability haplotypes estimated by PHASE were assigned to each study participant. If data were missing for any of the five polymorphic sites, the study participants were not included in the analysis. Each haplotype block can generate several possible haplotype pairs (diplotypes), and we also examined the diplotype effect. The diplotype data were treated as categorical variables and were incorporated as dummy variables in the logistic regression models.

To examine a possible interaction between *WRN* polymorphism and estrogen exposure, important pregnancy-related risk factors (the number of FTPs and estrogen exposure during the critical period between menarche and FFTP) were used as indices to estimate susceptibility to estrogen exposure. This is because the significant protection against the development of breast cancer, conferred by pregnancy, has been suggested to be due to the permanent differentiation of the vulnerable breast stem cells caused by pregnancy, thus reducing susceptibility to estrogen exposure (27, 28).

Results

We studied 935 female patients with pathologically confirmed infiltrating ductal carcinoma of the breast and 1,545 healthy female controls. The cases and controls had a similar average age (cases, 47.1 years; controls, 46.8 years). The proportion of cases with a positive family history of breast cancer in their first- and second-degree relatives was higher than that in controls (14.2% versus 11.2%, $P = 0.02$). In terms of reproductive risk factors associated with estrogen exposure, 64.1% of the cases experienced menarche before the age of 14 years compared with 68.1% of the controls, whereas only 39.2% of the cases had undergone the menopause compared with 49.8% of the controls. Using multiple logistic regression analysis, a significantly increased risk was found to be conferred by a positive family history of breast cancer in first- and second-degree relatives (yes versus no: adjusted OR, 1.36; 95% CI, 1.05-1.76). Having a history of FTP conferred significant protection against the development of breast cancer (yes versus no: adjusted OR, 0.84; 95% CI, 0.78-0.90). Risk was significantly increased by FTP-related risk factors, including being nulliparous (no history of FTP versus history of FTP: adjusted OR, 0.28; 95% CI, 0.02-3.22) and being older at FFTP (≧29 versus ≦23 years: adjusted OR, 0.83; 95% CI, 0.62-1.00; 24-28 versus ≦23 years: adjusted OR, 0.88; 95% CI, 0.72-1.09). No significant association was found between cancer risk and radiation exposure, hormone replacement therapy, smoking status, or body mass index (≧24 versus <24 kg/m^2: adjusted OR, 1.03; 95% CI, 0.85-1.24).

To define the tumorigenic contribution of the *WRN* gene, we examined whether the genotype distributions of SNPs in this gene were different between cases and controls. All five SNPs [rs10808311 (−17702T>C), rs11574218 (21826G>T), rs9649886 (46729A>C), rs2725362 (*Leu*^{1074}*Phe*, 83316T>G), and rs1346044 (*Cys*^{1367}*Arg*, 108690T>C)] were genotyped in cases and controls. The observed genotype frequencies for these five polymorphisms were all in Hardy-Weinberg equilibrium in the controls (data not shown). Logistic regression analysis, simultaneously considering well-known risk factors of breast cancer, showed that the rare homozygote CC of 46729A>C was significantly associated with a risk for breast cancer (adjusted OR, 1.48; 95% CI, 1.16-1.89; Table 1). To examine the haplotype effect, we examined more than one SNP to identify possible haplotypes of the *WRN* gene that would capture all of the contribution of the *WRN* locus to breast cancer. To do so, we used pairwise linkage disequilibrium to measure the linkage disequilibrium between pairs of SNPs, as indicated by the variable D', and obtained D' values of 1.00 between −17702T>C and 21826G>T, 0.00 between 21826G>T and 46729A>C, 0.84 between 46729A>C and *Phe*^{1074}*Leu*, and 0.59 between *Phe*^{1074}*Leu* and *Cys*^{1367}*Arg*. Thus, these SNPs (loci) fell into three haplotype blocks: block 1 (5′ block) composed of −17702T>C and 21826G>T, block 2 composed of 46729A>C and *Phe*^{1074}*Leu*, and block 3 composed of *Cys*^{1367}*Arg* (Table 1).

Each linkage disequilibrium block can generate up to four haplotypes, and their frequency distributions among cases and controls are shown in Table 2. For block 1, consisting of −17702T>C and 21826G>T, TG was the most common haplotype (54.9%) in our control group; the frequencies of the other three haplotypes in the controls are 38.8% (CT), 4.2%

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Table 1. Genotype frequencies of the *WRN* gene in breast cancer cases and controls and the adjusted OR for developing breast cancer

SNPs* genotypes	Cases (%)	Controls (%)	OR (95% CI)	Adjusted OR (95% CI)†	P	D'‡
rs10808311 (−17702T>C)						
TT	320 (34.3%)	542 (35.1%)	1.00	1.00		
TC	457 (49.0%)	740 (48.0%)	1.05 (0.87-1.25)	1.03 (0.90-1.17)	0.70	1.00
CC	155 (16.1%)	261 (16.9%)	1.00 (0.89-1.13)	1.02 (0.86-1.22)	0.81	
rs11574218 (21826G>T)						
GG	302 (32.3%)	513 (33.2%)	1.00	1.00		
GT	469 (50.2%)	735 (47.6%)	1.08 (0.90-1.30)	1.08 (0.95-1.24)	0.26	0.00
TT	164 (17.5%)	297 (19.2%)	0.97 (0.86-1.09)	0.94 (0.79-1.12)	0.47	
rs9649886 (46729A>C)						
AA	518 (55.8%)	860 (55.9%)	1.00	1.00		
AC	340 (36.6%)	596 (38.7%)	0.95 (0.80-1.13)	0.94 (0.83-1.07)	0.36	0.84
CC	71 (7.6%)	83 (5.4%)	1.19 (1.02-1.99)	1.48 (1.16-1.89)	0.002	
rs2725362 (*Leu*^{1074}*Phe*, 83316T>G)						
TT	382 (41.2%)	641 (41.7%)	1.00	1.00		
TG	438 (47.3%)	721 (46.8%)	1.02 (0.86-1.21)	1.01 (0.89-1.15)	0.87	
GG	107 (11.5%)	176 (11.5%)	1.02 (0.78-1.34)	1.03 (0.84-1.26)	0.77	0.59
rs1346044 (*Cys*^{1367}*Arg*, 108690T>C)						
TT	738 (79.2%)	1,225 (79.3%)	1.00	1.00		
TC	181 (19.4%)	300 (19.4%)	1.00 (0.82-1.23)	1.01 (0.82-1.25)	0.91	
CC	13 (1.4%)	19 (1.2%)	1.14 (0.56-2.31)	1.14 (0.53-2.46)	0.73	

*The SNP location is shown relative to the start codon ATG according to the National Center for Biotechnology Information genomic contig NT_07994.14. For SNPs in an exon, the position of the amino acid is also given. The ''rs'' number is the National Center for Biotechnology Information SNP cluster ID of each SNP.
† Adjusted OR in a multiple logistic regression model adjusted for age, family history of breast cancer, age at menarche, history of FTP, menopausal status, and body mass index.
‡ D' was calculated as a measure of the linkage disequilibrium between adjacent SNPs.

Table 2. Estimated haplotype frequencies of the *WRN* gene in breast cancer cases and controls and the significance of the association between harboring a specific haplotype and breast cancer development

Haplotypes*	Cases/controls	OR (95% CI)	Adjusted OR (95% CI) †	P
Block 1				
TG	1,041/1,699	1.00	1.00	
CT	738/1,201	1.00 (0.89-1.13)	1.00 (0.88-1.13)	0.99
TT	60/129	0.76 (0.55-1.04)	0.84 (0.54-1.32)	0.45
CG	33/63	0.86 (0.56-1.31)	0.78 (0.56-1.09)	0.15
Block 2				
AT	1,180/1,981	1.00	1.00	
CG	448/726	1.04 (0.90-1.19)	1.04 (0.90-1.20)	0.58
AG	205/347	0.99 (0.82-1.20)	1.00 (0.83-1.22)	0.99
CT	39/38	1.72 (1.10-2.71)	1.90 (1.20-3.02)	0.007

*Allele list for haplotype-tagging SNPs in 5′ to 3′ order. Block 1: rs10808311 (−17702T>C), rs11574218 (21826G>T). Block 2: rs9649886 (46729A>C), rs2725362 (*Leu*^{1074}*Phe*, 83316T>G).
†Adjusted OR in a multiple logistic regression model adjusted for age, family history of breast cancer, age at menarche, history of FTP, menopausal status, and body mass index.

(TT), and 2.0% (CG). None of these haplotypes was associated with breast cancer risk. For block 2, consisting of 46729A>C and *Phe*^{1074}*Leu*, AT was the most common haplotype (64.1%) in our control group; the frequencies of the other three haplotypes in controls are 23.5% (CG), 11.2% (AG), and 1.2% (CT). In this block, the CT haplotype was significantly associated with breast cancer risk (adjusted OR, 1.90; 95% CI, 1.20-3.02, P = 0.007).

Each haplotype block can generate several possible haplotype pairs (diplotypes), and the distributions of the diplotype frequencies in cases and controls are shown in Table 3. The diplotype of the most common haplotype (i.e., AT) of block 2 was selected as the reference in the diplotype analysis. The risk of breast cancer was estimated for each diplotype of *WRN* block 2 compared with that for the AT:AT diplotype, with adjustment for other covariates. For 46729A>C and *Phe*^{1074}*Leu*, the frequencies of the haplotype pairs CG/CT and CT/CT in breast cancer patients were significantly higher than those in controls. Our results, showing a significant association between risk of breast cancer and a specific locus (homozygote CC of 46729A>C) and the C-T haplotype of 46729A>C and *Phe*^{1074}*Leu*, suggest that *WRN* plays a significant role in the risk of breast cancer. To address the issue of multiple comparison, we used the Bonferroni correction. After these corrections, our results (Tables 1-3) remained the same, and all significant associations identified remained significant using the more stringent Ps.

Furthermore, we chose one SNP (46729A>C), which showed the most significant P in the multiple logistic regression analyses, to represent the allelic status of *WRN* to examine whether any association between *WRN* and breast cancer was modified by reproductive risk factors reflecting susceptibility to estrogen exposure. To exclude a false combination effect due to an unequal contribution of individual factors, we used a dummy variable coding scheme, with women who had no putative high-risk genotype and had been exposed to less estrogen (shorter menarche-FFTP interval) as the reference group. Multiple logistic regression analysis was used to estimate the multivariate adjusted ORs for each variable, and the risks associated with harboring a putative high-risk genotype, a risk factor, or both (joint effect) were estimated simultaneously. The results were consistent with the presence of an interaction ($P_{\text{interaction}}$ = 0.04), the highest risk being seen in women with both the reproductive risk factor (menarche-FFTP interval ≥16 years) and a putative high-risk genotype (*Vt/Vt*; adjusted OR, 3.89; 95% CI, 1.99-7.61). In addition, we estimated the risk associated with different haplotypes of *WRN*. The expected potential importance of a risk effect conferred by the at-risk haplotype of *WRN* was observed, but modification of this risk effect by the reproductive risk factor (longer menarche-FFTP interval) was supported by the findings shown in Table 4. The results were also consistent with the presence of a significant interaction between a high-risk haplotype and the reproductive risk factor ($P_{\text{interaction}}$ = 0.02). Within the putative high-risk haplotype stratum, there was a significant increase in risk of developing breast cancer with an increase in the menarche-FFTP interval (≥16 years; adjusted OR, 4.18; 95% CI, 1.53-9.44), whereas breast cancer risk associated with the high-risk haplotype was not significant in the stratum of women without the reproductive risk factor.

Finally, to confirm the joint effect between *WRN* genotypic polymorphism and reproductive risk factors and to investigate the potential importance of a protective effect of the number of FTPs in conjunction with *WRN*, we estimated the risk associated with the number of FTPs in women with variant genotypes. Consistent with the results shown in Table 4, significant interactions between *WRN* genotypic polymorphism and this risk factor were seen in analyses stratified by menopausal status ($P_{\text{interaction}}$ < 0.05), and a significantly reduced risk was restricted to women harboring homozygous or heterozygous wild-type genotypes (P_{trend} < 0.05; Table 5).

Discussion

To search for the mutator responsible for the genomic instability leading to breast tumorigenesis, in our previous studies (16-20), we attempted to identify specific genes and

Table 3. Distribution of *WRN* haplotype pairs in cases and controls and the risk (indicated by the adjusted OR) of developing breast cancer associated with harboring specific haplotype pairs

Haplotype pair		Cases/controls	OR (95% CI)	Adjusted OR (95% CI)*	P
Block 2 † of *WRN*					
AT	AT	369/625	1.00	1.00	
AT	CG	282/488	0.98 (0.81-1.19)	0.97 (0.79-1.19)	0.79
AG	AT	141/219	1.09 (0.85-1.40)	1.10 (0.85-1.42)	0.46
CG	CG	55/69	1.35 (0.93-1.97)	1.39 (0.94-2.05)	0.10
AG	CG	40/86	0.79 (0.53-1.17)	0.80 (0.53-1.21)	0.29
AG	AG	12/21	0.97 (0.47-1.99)	0.96 (0.46-2.04)	0.93
CG	CT	16/14	2.18 (1.07-4.43)	2.16 (1.02-4.56)	0.04
CT	CT	2/0	—	—	—
CG / CT	CT / CT (combined)	18/14	1.94 (0.93-4.02)	2.43 (1.17-5.03)	0.02
Other haplotype pairs		19/24	1.48 (0.81-2.70)	1.47 (0.78-2.76)	0.24

*Adjusted OR in a multiple logistic regression model adjusted for age, family history of breast cancer, age at menarche, history of FTP, menopausal status, and body mass index.
†Block 2: allele list for haplotype-tagging SNPs in 5′ to 3′ order: rs9649886 (46729A>C) and rs2725362 (*Leu*^{1074}*Phe*, 83316T>G).

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Table 4. Risk of breast cancer associated with the combination of *WRN* genotypes or haplotypes and a reproductive risk factor (interval between menarche and FFTP)

Genotypes/haplotype	Menarche-FFTP interval (y)*					
	≤9		10-15		≥16	
	Cases/ Controls	Adjusted OR (95% CI) †	Cases/ Controls	Adjusted OR (95% CI) †	Cases/ Controls	Adjusted OR (95% CI) †
rs9649886 (46729A>C)						
AA (*Wt/Wt*)	174/250	1.00	201/367	1.22 (0.90-1.64)	143/243	1.51 (1.06-2.16)
AC (*Wt/Vt*)	128/174	1.10 (0.80-1.51)	129/263	1.04 (0.75-1.43)	83/159	1.41 (0.94-2.11)
CC (*Vt/Vt*)	17/25	1.08 (0.56-2.09)	26/39	1.46 (0.83-2.58)	28/19	3.89 (1.99-7.61)
P_{trend} ‡		0.58		0.76		0.07
$P_{interaction}$§ = 0.04						
Haplotype block 2‖						
AT	415/564	1.00	448/858	1.18 (0.98-1.41)	317/559	1.41 (1.11-1.80)
AG	65/115	0.77 (0.54-1.09)	84/144	1.35 (1.03-1.76)	56/88	1.68 (1.12-2.52)
CG	153/213	1.02 (0.79-1.31)	164/323	1.25 (1.00-1.55)	13/190	1.79 (1.32-2.42)
CT	11/12	1.42 (0.64-3.47)	18/19	2.47 (1.39-4.37)	10/7	4.18 (1.53-9.44)
P_{trend} ‡		0.84		0.75		0.02
$P_{interaction}$§ = 0.02						

*The index is calculated as the age at FFTP minus the age at menarche in all parous women, the age at menopause minus the age at menarche in postmenopausal nulliparous women, and the age at breast tumor onset (case) or age at interview (control) minus the age at menarche in premenopausal nulliparous women.
†The adjusted OR for breast cancer development associated with the combination of *WRN* genotypes/haplotypes and a reproductive risk factor was estimated in a multivariate logistic regression model containing age, family history of breast cancer, number of FTP, menopausal status, body mass index, and a group of dummy variables to represent different statuses of *WRN* genotypes/haplotypes and reproductive risk factor (menarche-FFTP interval).
‡P_{trend} associated with the combined effect of genotype/haplotype and reproductive risk factor was estimated in a multiple logistic regression model, stratified by the interval (years) between menarche and FFTP, containing age, a family history of breast cancer, age at menarche, history of FTP, menopausal status, body mass index, and the number of putative high-risk allelotypes (in single SNP analysis) or the haplotypes coded as a continuous variable based on the adjusted OR associated with this haplotype.
§$P_{interaction}$ was calculated in a multiple logistic regression model containing age, family history of breast cancer, age at menarche, menopausal status, body mass index, the high-risk genotype/haplotype of *WRN*, reproductive risk factor (i.e., menarche-FFTP interval), and a (genotype/haplotype × reproductive risk factor) interaction term.
‖Haplotype block 2 is composed of genotypes of rs9649886 (46729A>C) and rs2725362 (*Leu*[1074]*Phe*, 83316T>G).

molecular mechanisms implicated in maintaining genomic stability. The rationale underlying this approach is based on the fact that instead of being a single-gene disease, cancer arises from aberrations in a complex, interconnecting network of multiple regulatory genes involved in normal growth control and differentiation processes. Given the high frequency of chromosomal abnormalities and mutations found in human cancers, the hypothesis that cancer is caused by a mutator phenotype was proposed (29) and has been confirmed by studying germline mutation of defective DNA repair genes causally leading to the initiation of hereditary cancer syndromes (30). The present study followed the same hypothesis and examined the breast tumorigenic contribution of *WRN*, a DNA helicase. Given the function of DNA helicases in many DNA metabolism pathways, including replication, DNA repair, recombination, transcription, and chromosome segregation (31-34), our hypothesis that *WRN* may serve as a mutator driving tumorigenesis is biologically reasonable. Genetic evidence that Werner syndrome cells show an increased frequency of genomic instability (31), and that the mouse model of Werner syndrome displays both accumulation of DNA damage foci and increased chromosomal instability (31, 32), provide support for the mutator role of *WRN.* Our finding that genotypic polymorphism of *WRN* was significantly associated with breast cancer risk is consistent with this hypothesis.

There is currently no evidence for genotype-phenotype associations for the SNPs of *WRN* examined in the present study, and how the risk is affected by these SNPs should therefore be interpreted with caution. It is possible that our epidemiologic findings may be explained by alternative mechanisms, such as (*a*) the polymorphisms may be in linkage disequilibrium with an exon change in the same gene that affects protein function; (*b*) intronic changes in gene sequences contain regulatory sequences, such as enhancers, which affect the level of expression through transcriptional regulation; (*c*) the SNPs in the gene may be linked to alterations in other adjacent unidentified genes, increasing breast cancer risk. To exclude the third possibility, we attempted to use more than one SNP in this gene to assign the haplotypes and to examine haplotype effects on cancer risk, and the information generated by haplotype analysis confirmed the contribution of *WRN*.

Table 5. Decreased risk (adjusted OR) of breast cancer development associated with the number of FTPs in women harboring different *WRN* genotypes, stratified by menopausal status

WRN, rs9649886 (46729A>C)	Adjusted OR (95% CI)*	
	AA and *AC*	CC
Premenopausal women		
No. FTPs		
0	1.00	1.00
≤2 children	0.63 (0.43-0.92)	0.66 (0.15-3.04)
≥3 children	0.58 (0.40-0.86)	0.39 (0.08-1.93)
P_{trend} †	0.02	0.17
$P_{interaction}$ ‡ = 0.04		
Postmenopausal women		
No. FTPs		
0	1.00	1.00
≤2 children	1.17 (0.65-2.11)	0.44 (0.06-3.34)
≥3 children	0.70 (0.40-1.21)	0.54 (0.10-2.92)
P_{trend} †	0.01	0.82
$P_{interaction}$ ‡ = 0.01		

*Adjusted OR of breast cancer development associated with the number of FTPs calculated in a multiple logistic regression model, containing age, family history of breast cancer, age at menarche, body mass index, and the number of FTPs.
†P_{trend} associated with the effect of the number of FTPs was estimated in a multiple logistic regression model containing age, a family history of breast cancer, age at menarche, body mass index, and the number of FTPs.
‡$P_{interaction}$ was calculated in a multiple logistic regression containing age, a family history of breast cancer, age at menarche, body mass index, the genotype of *WRN*, reproductive risk factor (i.e., the number of FTPs), and a (genotype × reproductive risk factor) interaction term.

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Although for a large gene, such as *WRN*, using more SNP coverage is a good idea in general, it may be not as informative as expected in our population because that Taiwanese population is relatively genetically homogenous; the linkage disequilibrium among SNPs is much stronger than that seen in other populations. The strength of the present study is that it is based on the candidate gene approach, examining the tumorigenic contribution of *WRN*, the germline mutation of which is causally linked to the development of Werner syndrome, an autosomal recessive disease characterized by premature aging, elevated genomic instability, and increase cancer incidence (6, 8). At the genetic level, our present finding that an increased risk of breast cancer was specifically associated with a homozygous variant allele of 46729A>C, an SNP located in the region encoding the COOH-terminal domain of WRN, but not with SNPs in the 5′-region (i.e., block 1), is of particular interest. WRN helicase has several major structural domains and forms complexes with other proteins involved both in cellular responses to DNA damage and in DNA replication (31, 32). Interestingly, all of these interacting proteins, such as FEN-1, PARP-1, polβ, Ku heterodimers, RAD52, p53 RPA, and polδ, bind to WRN via the COOH-terminal domain (amino acids 940-1432). It has been suggested that the COOH-terminal domain contains a unique region responsible for the protein-protein interactions of WRN (31, 32), and the results of our haplotype analysis showing that the frequencies of the *WRN* haplotype containing 46729A>C and $Phe^{1074}Leu$ in cases and controls were different, and that some haplotypes in this block of *WRN* were significantly associated with increased risk of breast cancer are consistent with this suggestion. Interestingly, another SNP ($Arg^{834}Cys$), located close to this haplotype block, is known to significantly reduce WRN helicase activity (35). However, the frequency of the minor allele of this SNP is very low (about 1%); thus, we did not examine its contribution. However, due to its location close to the COOH-terminal region, we cannot totally rule out the possibility that the significant risk associated with the haplotype block we defined was due to linkage disequilibrium between $Arg^{834}Cys$ and the SNPs in the COOH-terminal region.

If the *WRN* gene responsible for maintaining genome integrity were as important a cause of breast cancer formation as our results suggest, it is puzzling that, except for a recent study suggesting *WRN* may act as a low-penetrance risk factor in familial breast cancer development (36), there is no genetic evidence linking breast cancer and mutated *WRN*. We previously proposed an explanation for this paradox and suggested that the probability of manifesting the tumorigenic phenotype might depend on a joint effect between these polymorphic alleles and endogenous or exogenous risk factors (17, 20, 21). For breast cancer development, we suspect that prolonged estrogen exposure (indicated by early menarche and late menopause) or higher susceptibility to estrogen exposure (indicated by no history of FTP, fewer FTPs, or older age at FFTP) may be one factor. In addition, a long menarche-FFTP interval naturally creates a large estrogen window favorable for tumor induction by hormonal mechanisms (14). Based on this hypothesis, our study evaluated the combined effect of genetic polymorphisms and reproductive risk factors and examined whether breast tumorigenesis due to the *WRN* gene was influenced by a reproductive factor. Our findings in Table 4 are consistent with the idea that the high-risk genotypes/haplotypes of *WRN* and an estrogen-related risk factor act jointly to increase breast cancer risk. In women exposed to a greater cumulative amount of estrogen (due to a longer menarche-FFTP interval), the breast epithelium might have experienced more mitogenic stimulation and subsequently would have a higher potential to develop breast cancer if the cells have a suboptimal capacity to maintain genomic stability because of a variant *WRN*. An interaction between *WRN* and a reproductive risk factor in determining breast cancer risk was also shown by the findings in Table 5, in which the protective effect resulting from having a higher number of FTP was not the same in women harboring different genotypes of *WRN*, being more obvious in those having wild-type genotypes.

On the basis of a candidate gene approach and a relatively large sample size, the present study supports the idea of a breast tumorigenic contribution of *WRN*, a gene involved in critical mechanisms maintaining chromosomal stability. Recently, the SNP $Cys^{1367}Arg$ of *WRN* has been suggested to act as a low-penetrance risk factor in determining familial breast cancer risk in German familial breast cancer patients (36). However, in the present study, we examined the risk associated with this SNP but did not confirm its contribution, which may reflect racial differences in SNPs in different populations. We were therefore more interested in the functional interaction between WRN and ATM that has recently been shown, with WRN being phosphorylated by an ATM-dependent pathway in response to DNA damage (37). Because *ATM* is a well-documented breast cancer susceptibility gene (38), this association between WRN and ATM provides additional support for the tumorigenic contribution of *WRN* to breast cancer development. The advantages of the present study include the high frequencies of the SNPs and the large sample size. The validity of our findings is supported by the consistent results obtained in genotype, haplotype, and diplotype analyses. Thus, despite minor limitations, such as a hospital-based study design and the requirement of using more tagging SNP coverage, the results of this study provide new support for the tumorigenic role of *WRN* in breast cancer development, suggesting that breast cancer might be driven by aging associated with variant *WRN*, the tumorigenic contribution of which could be enhanced as a result of increased cell growth due to estrogen exposure.

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